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Center for Veterinary Biologics
Testing Protocol**

SAM 604

Supplemental Assay Method for the Evaluation of Koch's Old Tuberculin

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Supplemental Assay Method for the Evaluation of Koch's Old Tuberculin

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Supplemental Assay Method for the Evaluation of Koch's Old Tuberculin

1. Introduction

This is a Supplemental Assay Method (SAM) procedure for the evaluation of production lots of Koch's old tuberculin in accordance with the Code of Federal Regulations, Title 9 (9 CFR), Part 113.406.

2. Materials

2.1 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand names listed below.

2.1.1 *Mycobacterium tuberculosis* reference tuberculin, current lot. This reference is obtained from the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Veterinary Services (VS), Center for Veterinary Biologics (CVB).

2.1.2 *M. tuberculosis* sensitizing agent, current lot. This reagent is available from the CVB.

2.1.3 Saline solution, 0.85%, pH 6.4

2.1.4 Validated digital calipers or metric ruler made of clear plastic

2.1.5 Needles, 20-gauge x 1-inch and 26-gauge x 3/8-inch

2.1.6 Disposable syringes, 1-mL and 3-mL

2.1.7 Pipettes, 1-mL, 5-mL and 25-mL

2.1.8 Glass serum bottles, 10-mL and 125-mL

2.1.9 Rubber seals and metal caps for serum bottles

2.1.10 Crimper for aluminum seals

2.1.11 Depilatory cream

2.1.12 Animal clippers, equipped with a sharpened #40 blade, or equivalent

2.1.13 Ear tags for small animals

2.1.14 Cage cards

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2.2 Animals

Guinea pigs, 500-700 g, white-haired, nonpregnant females. Ten guinea pigs are required for each lot to be tested. Two additional guinea pigs are required as nonsensitized controls. All guinea pigs used for a test must be from the same source and housed and fed in the same manner.

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel must have working knowledge of the use of general laboratory chemicals, equipment, and glassware and have specific training and experience in the safe handling of laboratory animals.

3.2 Selection and handling of test animals

3.2.1 Select guinea pigs that are healthy, free of external parasites, and have an unblemished hair coat.

3.2.2 Examine guinea pigs the day they are received, and house according to standard operating procedures.

3.2.3 When the test is concluded, instruct the animal caretakers to euthanize the guinea pigs, unless they can be used as second use animals for additional testing or blood collection.

3.3 Preparation of reagents

3.3.1 Saline solution, 0.85% (NVSL Media #30201)

Sodium chloride	8.5 g
Water q.s. to	1.0 L

Adjust pH to 6.4. Autoclave at 121°C for 15 minutes. Store at 20°- 25°C for no longer than six months.

3.4 Preparation of supplies

3.4.1 Sterilize all glassware before use.

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3.4.2 Use only sterile supplies (syringes, needles, rubber seals, metal caps, etc.).

3.5 Test animal sensitization

3.5.1 Sensitize 10 guinea pigs per lot of tuberculin to be evaluated. Wait **30 to 120 days** before performing the potency portion of the assay.

3.5.2 Administer 0.5 mL of *M. tuberculosis* sensitizing agent intramuscularly to each guinea pig. Split the dose, administering 0.25 mL into each rear leg. Use 3-mL syringes fitted with 20-gauge x 1-inch needles. Identify the sensitized guinea pigs by cage cards or equivalent.

3.5.3 Retain 2 guinea pigs as nonsensitized controls.

4. Performance of the Potency Test

4.1 Preparation of guinea pigs for the potency assay

Clip the abdomen of each guinea pig with animal clippers. Generously apply a depilatory cream to the clipped abdomen. Wait at least 3 minutes. Wash off the depilatory cream with warm water within 10 minutes of application and dry the abdomen with a soft towel. Allow the guinea pigs to rest for a minimum of 4 hours before administering the tuberculin injections.

4.2 Preparation of tuberculin dilutions

4.2.1 Preparation of control dilutions

1. Vortex the bottle several times to mix. Remove 0.5 mL of reference tuberculin from the bottle using a 1-mL tuberculin syringe. Add tuberculin to a 10-mL serum bottle containing 1.5 mL saline, to create a 1:4 dilution. Cap the bottle, label, and mix well by vortexing.

2. Add 1 mL of the 1:4 dilution of tuberculin, measured in a 1-mL tuberculin syringe, to a 10-mL serum bottle containing 1.5 mL of saline to create a 1:10 dilution. Cap the bottle, label, and mix well by vortexing.

3. Repeat Steps 1 and 2 with the lot of tuberculin to be tested.

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4.2.2 Preparation of assay dilutions

Note: Prepare dilutions of the reference tuberculin and the lot to be tested in exactly the same manner.

1. Dispense 99 mL of saline into a 125-mL serum bottle. Add 1.0 mL of reference tuberculin, measured in a 1-mL tuberculin syringe. This is a 1:100 dilution. Cap and label the bottle. Mix well by vortexing.
2. Label 2, 10-mL serum bottles 1:200 and 1:400, respectively. Use a 5-mL pipette to add 5.0 mL of saline to each of the bottles labeled 1:200 and 1:400.
3. Use a 5-mL syringe to add 5.0 mL of the 1:100 dilution prepared in Step 1 into the bottle labeled 1:200. Close the bottle with a rubber stopper. Mix well by vortexing.
4. Use a 5-mL syringe to transfer 5.0 mL of the 1:200 dilution into the bottle labeled 1:400. Close the bottle with a rubber stopper. Mix well by vortexing.
5. Clamp aluminum seals over the rubber stoppers on the 3 serum bottles containing the diluted tuberculin.
6. Repeat Steps 1-5 with each lot of tuberculin to be tested.

4.3 Intradermal injection of test animals

4.3.1 Identify 6 injection sites on the abdomen of each sensitized guinea pig, with 3 sites on each side, evenly spaced, equidistant from the midline. (See sample worksheet in the **Appendix**.) Do not mark the sites on the abdomen with ink.

4.3.2 Each sensitized guinea pig will receive a total of 6 injections, 1 at each previously identified site as recorded on the worksheet. Each preparation (1:100, 1:200, or 1:400 dilution of the reference tuberculin; or 1:100, 1:200, or 1:400 dilution of the test tuberculin) will be injected into each site.

4.3.3 Administer 0.05 mL of each preparation at the sites by intradermal injection using the full length of the needle to prevent leaking of the product. Needles must be sharp and it is recommended that they are replaced after insertion through a rubber stopper or after injecting 10 guinea pigs. Use 1-mL tuberculin syringes fitted with 26-gauge x 3/8-inch needles.

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4.3.4 Identify 4 injection sites on each nonsensitized (control) guinea pig, equidistant, 2 on each side of the midline. (See sample worksheet in the **Appendix**.) Do not mark the sites on the abdomen with ink.

4.3.5 Each nonsensitized (control) guinea pig will receive a total of 4 injections, 1 at each previously identified site. Each preparation (1:4 or 1:10 dilution of the reference tuberculin; or 1:4 or 1:10 dilution of the test tuberculin) will be injected into each site.

4.3.6 Administer 0.05 mL of each preparation by intradermal injection at the sites determined on the sample worksheet in the **Appendix**. Use 1-mL tuberculin syringes fitted with 26-gauge x 3/8-inch needles.

4.3.7 Guinea pigs are identified by small animal ear tags and cage cards.

5. Interpretation of the Test Results

5.1 Recording of test results

5.1.1 Measure the test reactions at 24 hours following injection.

5.1.2 Measure the greater and lesser diameters of erythema and/or swelling to the closest mm at each injection site. Gently palpate the lesion to determine the margin of the swelling, which may or may not extend beyond the margin of erythema. Record the results on the worksheet .

5.1.3 Calculate the area of erythema and/or swelling (in mm²) by multiplying the greater and lesser diameter measurements.

5.1.4 For each dilution of tuberculin tested, add together the reaction areas for that dilution from each of the 10 guinea pigs. Record the sum reaction for each dilution on the test record.

5.1.5 Add together the sums obtained for each dilution of the reference tuberculin to generate the grand total for the reference tuberculin. Similarly calculate a grand total for the test serial. Record these results on the test record.

5.1.6 Divide the grand total for the test serial by the grand total for the reference tuberculin. Multiply by 100 to express the test serial reaction as a percentage of the reference tuberculin reaction. Record all calculations on the test record.

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5.2 Criteria for a valid test

5.2.1 The control guinea pigs must have no signs of reaction at 24 hours.

5.2.2 For a satisfactory serial, the reaction of the test serial must be 25% of the reaction of the reference tuberculin, i.e., the percentage calculated in **Section 5.1.6** must fall between 75% and 125%.

6. Reporting of Test Results

Report results of the test(s) as described by standard operating procedures

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.406, U.S. Government Printing Office, Washington, DC.

7.2 History of reagents: The *M. tuberculosis* sensitizing agent is a moist, heat-killed preparation of *M. tuberculosis* strains Pn, C, and DT. These strains were originally obtained from Cooper Animal Health. *M. tuberculosis* reference tuberculin was obtained from the USDA, APHIS, VS, NVSL, Ames, Iowa 50010.

8. Summary of Revisions

- The Contact has been changed from Charles Egemo to Janet Wilson.
- **2.1.4:** The phrase "Validated digital calipers" has been added,
- **2.1.7:** One-mL pipettes have been added to the list of equipment.
- **2.1.12:** The phrase "equipped with a sharpened #40 blade, or equivalent" has been added.
- **2.1:** "Ear tags for small animals" and "Cage cards" have been added to the list of reagents and supplies.
- **3.2:** This section has been revised to reflect current practices.
- **3.3.1:** The phrase "Store at 20°- 25°C for no longer than six months" has been added.
- **4.1/4.2/4.3:** These sections have been revised to reflect current practices.

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- **5.1:** This section has been revised to reflect current practices.

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Appendix

Koch's Old Tuberculin Test Record

Tuberculin ID _____ Date read _____ Initials _____
 Serial Dilution 1 = 1:100 Serial Dilution 2 = 1:200 Serial Dilution 3 = 1:400 Reference Dilution 4 = 1:100 Reference Dilution 5 = 1:200 Reference Dilution 6 = 1:400

Guinea Pig Tag#	Site A Reference	Site B Test Serial	Site C Reference	Site D Test Serial	Site E Reference	Site F Test Serial
	4	1	5	2	6	3
	6	3	5	2	4	1
	5	2	4	1	6	3
	5	2	6	3	4	1
	6	3	4	1	5	2
	6	3	5	2	4	1
	4	1	5	2	6	3
	4	1	6	3	5	2
	5	2	4	1	6	3
	5	2	6	3	4	1

Total of Serial Site B _____ mm² Total of Serial Site D _____ mm² Total of Serial Site F _____ mm²

Grand Total of Serial : _____ mm²

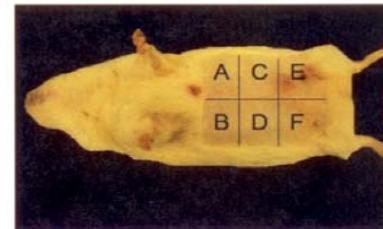
Total of Reference Site A _____ mm² Total of Reference Site C _____ mm² Total of Reference Site E _____ mm²

Grand Total of Reference: _____ mm²

Percent Response = $\frac{\text{Grand total test Serial}}{\text{Grand total Reference tuberculin}} \times 100 = \text{_____} \%$

Satisfactory serial = The reaction of the test serial must be 25% of the reaction of the reference tuberculin. The percentage calculated must fall between 75% and 125%.

NR= No reaction Initials _____ Date _____



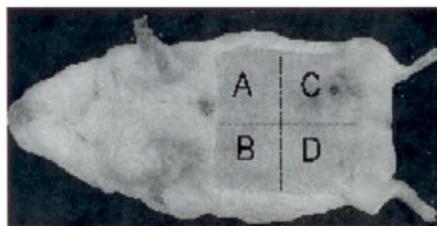
Sensitized guinea pig

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Koch's Old Tuberculin Test Record

Tuberculin ID _____ Date read _____ Initials _____
 Serial Dilution 1 = 1:4 Serial Dilution 2 = 1:10 Reference Dilution 3 = 1:4 Reference Dilution 4 = 1:10

Guinea Pig Tag#	Site A Reference	Site B Test Serial	Site C Reference	Site D Test Serial
	3	1	4	2
	4	2	3	1



Non-Sensitized Control guinea pig

The control guinea pigs must have no signs of reaction at 24 hours for a valid test.

NR= No reaction

Initials _____ Date _____

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